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Chromosomal and Ti plasmid characterization of tumorigenic strains of three *Agrobacterium* species isolated from grapevine tumours

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Fifty-six tumorigenic Spanish grapevine strains of *Agrobacterium* spp. were tested for biovar classification, pathogenicity on several hosts, opine utilization, 16S rRNA gene sequencing and PCR amplifications using five primer sets targeting chromosomal and Ti plasmid genes. Fifty of them belonged to *A. vitis* (biovar 3), three to *A. tumefaciens* (biovar 1) and three to *A. rhizogenes* (biovar 2). All strains were tumorigenic on grapevines. Most *A. vitis* strains were also pathogenic on tomato and tobacco plants, while the three *A. tumefaciens* strains were only pathogenic on grapevine. Although most *A. vitis* strains used octopine, 12 utilized neither octopine nor nopaline. 16S rRNA gene sequencing clearly distinguished between strains belonging to the three species. Those of *A. vitis* could be further divided into three chromosomal backgrounds according to their 16S ribosomal RNA gene sequences. No universal primer pair was found for the detection of all three *Agrobacterium* species isolated from grapevine. DNA from all *A. vitis* strains was amplified with the chromosomally-encoded pehA primer pair. In both *A. vitis* and *A. tumefaciens* a correlation was observed between the amplifications obtained using the tmr and the virA Ti-plasmid-targeting primer pairs. Three types of Ti plasmid were found in *A. vitis* strains according to their PCR amplifications and opine utilization profiles. A given chromosomal background harboured only one type of Ti plasmid within the strains from each analysed sample, showing a strong association between chromosomal backgrounds and Ti plasmids in *A. vitis*.

Keywords: biovars, crown gall, opines, PCR, 16S rRNA gene sequencing, *Vitis vinifera*

Introduction

Crown and cane galls are among the most important and widespread bacterial diseases of grapevines (*Vitis vinifera*) (Burr & Otten, 1999). Gall development relies on the transfer to the plant cell of a DNA (T-DNA) fragment from the tumour-inducing (Ti) plasmid of *Agrobacterium*. Once transferred to the plant cell, the T-DNA integrates into the plant nuclear genome and T-DNA genes are transcribed. Genes located on T-DNA fall into two groups: one including genes encoding phytohormone biosynthesis, which are responsible for tumour development, and the second encoding enzymes catalysing the biosynthesis of low-molecular-weight compounds, called opines (reviewed in Zhu *et al*., 2000 and Zupan *et al*., 2000). These tumour-specific compounds play a key role in the ecology of the *Agrobacterium*-plant interactions (reviewed in Dessaux *et al*., 1998).

The genus *Agrobacterium* can be divided into at least three different clusters that correspond to biovars 1, 2 and 3, as defined by Keane *et al*. (1970). Biovars 1 and 2 possibly define different species, referred to in this work as *A. tumefaciens* and *A. rhizogenes*, respectively. Biovar 3 is regarded as the *A. vitis* species, which includes strains isolated only from grapes (Ophel & Kerr, 1990). Grapevine galls are predominantly caused by *A. vitis* worldwide and they may have mainly octopine/cucumopine-, nopaline- or, less frequently, vitopine-type Ti plasmids (Paulus *et al*., 1989; Burr *et al*., 1998; Burr & Otten, 1999; Ridé *et al*., 2000). Occasionally, strains of *A. tumefaciens* (biovar 1) with octopine/cucumopine or nopaline Ti plasmids may also occur in grapevine (Knauf *et al*., 1983;

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Ridé *et al*., 2000; Argun *et al*., 2002; Szegedi *et al*., 2005). More rarely, *A. rhizogenes* (biovar 2) strains have been reported from grapevine tumours (Panagopoulos *et al*., 1978; Süle, 1978), but they were not well characterized.

The most serious outbreaks of crown and cane galls of grapevine are usually recorded in cool climate regions, but the disease has also been reported from the Mediterranean region (Burr *et al*., 1998). The incidence of the disease and its importance in vineyards is increasing in Mediterranean and nearby countries, such as Israel (Haas *et al*., 1991), Iran (Mohammadi & Fatehi-Paykani, 1999), France (Ridé *et al*., 2000), Turkey (Argun *et al*., 2002) and Spain (Lastra, 1998; López, 2004). However, no systematic collection, differentiation and characterization of *Agrobacterium* spp. strains isolated from Spanish vineyards have yet been performed. The economic losses caused by this disease are not only related to intrinsic damage in infected plants, but also to the prohibition of commercial use of plants with tumours (Anonymous, 1993). This requires rapid, sensitive and specific methods to diagnose *Agrobacterium* in plant material, possibly using PCR-based techniques (Cubero *et al*., 1999; López *et al*., 2003). Thus, better characterization of *Agrobacterium* strains from grapevine would facilitate the selection of the most accurate protocols for routine detection and subsequent control of the sanitary status of plants (Burr *et al*., 1998; Ridé *et al*., 2000). To this end, 56 pathogenic strains were collected from grapevine tumours in vineyards cultivated in different areas of Spain and characterized according to their Ti-plasmid-borne and chromosome-encoded traits.

Materials and methods

Collection of *Agrobacterium* strains

Samples from various grape cultivars were taken from six Spanish provinces between 1997 and 2006. Trunks and canes showing crown or aerial galls were analysed. Before performing isolations, the tumours were washed with soapy water and rinsed, flamed, and the epidermis removed with a sterile scalpel. Selected pieces were crushed in 4 mL sterile distilled water and, after 30 min, 50 μL of tissue macerates were streaked on MG-L medium (Cangelosi *et al*., 1992) and on the semiselective media for *Agrobacterium* biovar 1 (Schroth *et al*., 1965), biovar 2 (New & Kerr, 1971) and biovar 3 (Roy & Sasser, 1983). After incubation for 3–5 days at 25°C, one to 10 colonies from each tumour were selected on the basis of their morphology and purified twice on MG-L medium. Colonies morphologically resembling *Agrobacterium* were preliminary submitted to some tests to differentiate *Agrobacterium* spp; (Gram reaction, glucose metabolism, urease production and aesculin-β-glucosidase activity) according to Moore *et al*. (1988). One to 10 *Agrobacterium* strains from each sample, from different plants or tumours, were used for further characterization and stored at –80°C in 30% glycerol.

Pathogenicity assays

The plant species used for tumorigenic assays were tomato (*Solanum lycopersicum* formerly *Lycopersicon esculentum*), tobacco (*Nicotiana tabacum*) and grape (*Vitis vinifera*) cv. Tempranillo. Two 4-week-old tomato and tobacco seedlings were each stab-inoculated at two different points in the stems with a needle touched onto 48-h-old bacterial cultures grown on MG-L solid medium. Similarly, green shoots of grape plants were stem-inoculated at two internodes. Two or more replicate plants of each species were inoculated with each strain. A further host-range study was performed on datura (*Datura stramonium*), bean (*Phaseolus vulgaris*) and aubergine (*Solanum melongena*) with selected strains. Several well-known strains of different species were inoculated as positive and negative reference controls. Inoculated plants were maintained in a greenhouse at about 25°C. Appearance of tumours was visually assessed 6 weeks after inoculation in herbaceous hosts and after 3 months in grape plants.

Biovar determination

For biovar determination, the following tests were performed according to Moore *et al*. (1988) and Cubero & López (2001): citrate utilization; growth and pigmentation in ferric ammonium citrate; alkali production from malonic, L-tartaric and mucic acids; acid production from sucrose, melezitose and erythritol; tolerance to 2% NaCl and 3-ketolactose production. Well-characterized *Agrobacterium* strains belonging to the three biovars were included as reference strains.

Opine utilization assays

The utilization of octopine and nopaline was analysed in microtitre plates as described by López *et al*. (1988). Well-characterized *Agrobacterium* strains were used as nopaline and octopine reference control strains.

16S rRNA gene sequencing

Amplification of partial (*c*. 500 bp) or almost complete (*c*. 1250 bp) sequences of the 16S rRNA gene was performed as described by Martínez-Murcia *et al*. (1999). DNA sequences were determined by direct sequencing of the PCR product on an ABI 3100 Avant sequencer (Applied Biosystems). The phylogenetic tree was inferred using a neighbour-joining method. Bootstrap analysis was based on 1000 resamplings.

PCR amplifications

Bacterial suspensions in sterile distilled water were prepared from 48-h-old cultures on MG-L solid medium and adjusted to *c*. 10^8 colony forming units (CFU) mL^{-1} (OD_{600} = 0·1). DNA was extracted using the Easy-DNA™ kit (Invitrogen) according to the manufacturer's

Table 1 Oligonucleotide primers used to characterize *Agrobacterium* spp. strains isolated from grapevine

Name	Gene target	Replicon specificity	Source
pehA	Pectin enzyme hydrolase	Biovar 3 chromosome	Eastwell *et al.*, 1995
virA	Virulence sensor gene	Limited host-range Ti plasmids	Eastwell *et al.*, 1995
FGP tmr 530/ FGP tmr 701'[a]	Cytokinin biosynthesis, Tumour formation gene (*tmr*)	Nopaline- and octopine-type Ti plasmids	Nesme *et al.*, 1989
virB11/virG15'[b]	Intercistronic region between *vir*B and *vir*AG operons	Nopaline-type Ti plasmid	Nesme *et al.*, 1989
VCF/VCR	Virulence *virC* operon	All types of Ti plasmid	Sawada *et al.*, 1995

[a]Referred to in the text as tmr primers.
[b]Referred to in the text as virB/G primers.

instructions and used as template. Ti-plasmid-less strain NT1 and the non-pathogenic strain K84, as well as sterile water, were included in every DNA preparation set as negative controls. PCR amplifications were conducted with five primer pairs previously reported for amplification of either chromosomal or plasmid-borne target genes of *Agrobacterium* spp., following the reaction conditions originally described by the respective authors. Name, gene target, replicon specificity and sources for the different primers used are indicated in Table 1. Amplified products were analysed by 2% agarose gel electrophoresis, and DNA fragments were visualized under UV light after staining with ethidium bromide. Amplifications were repeated at least twice. Several pathogenic and non-pathogenic strains harbouring different types of Ti plasmids were used as positive and negative reference controls.

Results

Biovar affiliation and pathogenicity of *Agrobacterium* spp. strains

The 56 selected strains were Gram-negative, utilized glucose only in aerobiosis, showed urease activity and degraded aesculin. They were further characterized by pathological, biochemical and molecular traits (Table 2). The majority of the strains (50 out of 56) were initially classified by biochemical tests as biovar 3 (*A. vitis*). The strain isolated from sample 2680 was negative for citrate utilization while the other *A. vitis* strains were positive. As expected, most of the biovar 3 strains were isolated on semiselective biovar 3 Roy and Sasser's medium. Two strains from sample 2709 and the strain isolated from sample 316 were grown on Schroth's medium and further classified as biovar 1. However, they utilized tartrate in contrast to typical biovar 1 strains (Table 2). Surprisingly, in sample 2709, tartrate-utilizing biovar 1 and -3 strains were found together and isolated from the same tumours (Table 2). Also, three strains from sample 1698 isolated on New & Kerr's medium were further classified as biovar 2 (Table 2).

All *Agrobacterium* spp. strains studied in this work induced tumours on grapevine plants (Table 2). All *A. vitis* strains induced tumours on tobacco plants, and all but three on tomato plants. Three selected *A. vitis* strains did not induce tumours on bean or aubergine, but they were tumorigenic on datura (data not shown). The three *A. tumefaciens* strains did not induce tumours either on tomato or tobacco plants (Table 2). Further inoculation studies confirmed the limited host range of these *A. tumefaciens* strains from grape, because they were not able to produce tumours on datura, bean or aubergine. In contrast, the reference strain of *A. tumefaciens* induced tumours in those hosts (data not shown). The three *A. rhizogenes* strains were pathogenic on tomato and tobacco, in addition to grape.

Opine utilization

Most of the *A. vitis* strains (35 out of 50) utilized octopine, but not nopaline, 12 strains did not utilize either of them and the three from sample 2739 utilized both opines. The three *A. tumefaciens* strains isolated from grape utilized octopine, but not nopaline, and the three *A. rhizogenes* strains utilized nopaline, but not octopine (Table 2).

16S rRNA gene sequencing

A dendrogram (Fig. 1) was constructed to display the relationship of partial 16S rRNA gene sequences of selected *Agrobacterium* strains belonging to the three species found on grapevine tumours. 16S rRNA gene sequences clearly differentiated strains belonging to the three different species, reinforcing their initial biovar classification. The three *A. tumefaciens* strains presented the same partial 16S rRNA gene sequence with 100% similarity to that of *A. tumefaciens* LMG 196 and CFBP 2714 strains from the database (sequence named Bv.1). The three *A. rhizogenes* strains and the reference biovar 2 strain IVIA 436-3 presented the same partial 16S rRNA gene sequence with 100% similarity to that of the *A. rhizogenes* strain LMG950 from the database (sequence named Bv.2). However, among the 50 *A. vitis* strains, three different partial 16S rRNA gene sequences were found (sequences named Bv. 3 types A, B and C). Among them, 14 strains and the reference biovar 3 strain IVIA 339-26 had sequence type A, 20 strains had sequence type B and 16 had sequence type C (Table 2). The three different types of

Table 2 Origin and pathological, biochemical and molecular traits of *Agrobacterium* spp. strains isolated from grapevine tumours

Plant sample code	Province	No. of strains	Strain code	Species (biovar)	Tumour induction			Tartrate utilization	Opine utilization		16S rRNA gene sequence type[a]	PCR profile				
					Tomato	Tobacco	Grape		Octopine	Nopaline		pehA	virA	tmr	VirB/G	VCF/VCR
2739	Albacete	3	IVIA 2739-16[b] IVIA 2739-17 IVIA 2739-18	*A. vitis* (3)	+	+	+	+	+	+	Bv.3 (C)	+	+	+	–	–
		2	IVIA 2739-29[c] IVIA 2739-35	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
2979	Albacete	1	IVIA 2979-10[d]	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
3105	Albacete	4	IVIA 3105-8a IVIA 3105-9b IVIA 3105-10a IVIA 3105-5c (25)[e]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
3112	Albacete	1	IVIA 3112-A2-2a[f]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
1698	Badajoz	3	IVIA 1698-2b-2[g] IVIA 1698-2b-3 IVIA 1698-2b-6	*A. rhizogenes* (2)	+	+	+	+	–	+	Bv.2	–	–	+	+	+
2709	Ciudad Real	8	IVIA 2709-1a-1-1 IVIA 2709-1a-1-2 IVIA 2709-2a-4-1[h] IVIA 2709-2b-2 IVIA 2709-2c-4-2 IVIA 2709-2d-1 IVIA 2709-2d-2 IVIA 2709-2d-3-1	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
		2	IVIA 2709-2b-1-2[i] IVIA 2709-2b-2-1	*A. tumefaciens* (1)	–	–	+	+	+	–	Bv.1	–	+	+	–	–
103/05	Ciudad Real	1	CITA 103/05-Av4[j]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (A)	+	–	–	–	–
		1	CITA 103/05-Av5	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
2680	Huesca	1	IVIA 2680-2-a3[k]	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
6/04	Huesca	1	CITA 6/04-Av2[j]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
3001	Madrid	3	IVIA 3001-2-G[l] IVIA 3001-3-1-Ha IVIA 3001-3-2-Ha	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
		3	IVIA 3001-3-Ba[m] IVIA 3001-6-6-Aa IVIA 3001-6-7-Aa	*A. vitis* (3)	–	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–

Table 2 *Continued*

Plant sample code	Province	No. of strains	Strain code	Species (biovar)	Tumour induction			Tartrate utilization	Opine utilization		16S rRNA gene sequence type[a]	PCR profile				
					Tomato	Tobacco	Grape		Octopine	Nopaline		pehA	virA	tmr	VirB/G	VCF/VCR
2811	Murcia	1	IVIA 2811-1a[n]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (C)	+	+	+	–	–
84	Orense	1	USC 84-334[o]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (A)	+	+	+	–	–
184	Orense	1	USC 184-472[p]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (C)	+	+	+	–	–
194	Orense	1	USC 194-505[q]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
266	Orense	1	USC 266-271[r]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
273	Orense	1	USC 273-165[s]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (B)	+	+	+	–	–
274	Orense	1	USC 274-426[t]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (A)	+	+	+	–	–
314	Orense	1	USC 314-229[u]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (C)	+	+	+	–	–
316	Orense	1	USC 316-257[v]	*A. tumefaciens* (1)	–	–	+	+	+	–	Bv.1	–	–	–	–	–
52/04	Teruel	1	CITA 52/04-Av3[w]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (C)	+	+	+	–	–
44/06	Zaragoza	6	CITA 44/06-Av18[x]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (C)	+	+	+	–	–
			CITA 44/06-Av20													
			CITA 44/06-Av22													
			CITA 44/06-Av24													
			CITA 44/06-Av25													
			CITA 44/06-Av27													
		2	CITA 44/06-Av12[y]	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
			CITA 44/06-Av14													
129/05	Zaragoza	1	CITA 129/05-Av6[z]	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (A)	+	–	–	–	–
153/05	Zaragoza	1	CITA 153/05-Av8[a′]	*A. vitis* (3)	+	+	+	+	–	–	Bv.3 (C)	+	–	–	–	–
240/05	Zaragoza	2	CITA 240/05-Av9[b′]	*A. vitis* (3)	+	+	+	+	+	–	Bv.3 (C)	+	+	+	–	–
			CITA 240/05-Av10													
Reference strains																
C58				*A. tumefaciens* (1)	+	+	+	–	–	+	n.d.	–[e′]	–	+	+	+
NT1				*A. tumefaciens* (1)	–	–	–	–	–	–	n.d.	–[e′]	–	–	–	–
B6				*A. tumefaciens* (1)	+	+	+	–	+	–	n.d.	–	–	+	–	+
436-3			IVIA 436–3′NA[c′]	*A. rhizogenes* (2)	+	+	+	+	–	+	Bv.2	–	–	+	+	+
K84				*A. rhizogenes* (2)	–	–	–	+	n.d[f]	n.d.	n.d.	–	–	–	–	–
339-26			IVIA 339-26[d′]	*A. vitis* (3)	+	+	+	+	+	+	Bv.3 (A)	+	+	–	–	–

[a]Partial amplification of the 16S rRNA gene (*c.* 500 bp) of *A. vitis* (biovar 3) strains, classified into types A, B and C as described in the text and shown in Fig. 1.

[b–d′]Accession numbers of the 16S rRNA gene sequence deposited in GenBank database: [b]EF590291; [c]EF590292; [d]EF590294; [e]EF590297; [f]EF590298; [g]EF213641; [h]EF590289; [i]EF590290; [j]EF590301; [k]EF590288; [l]EF590299; [ll]EF590295; [m]EF590296; [n]EF590293; [o]EF590307; [p]EF590308; [q]EF590309; [r]EF590310; [s]EF590311; [t]EF590312; [u]EF590313; [v]EF590314; [w]EF590300; [x]EF590305; [y]EF590306; [z]EF590302; [a′]EF590303; [b′]EF590304; [c′]EF590315; [d′]EF590316.

[e′]Amplification fragment (*c.* 500 bp) different from that expected was obtained when using pehA primers.

[f]n.d.: not determined.

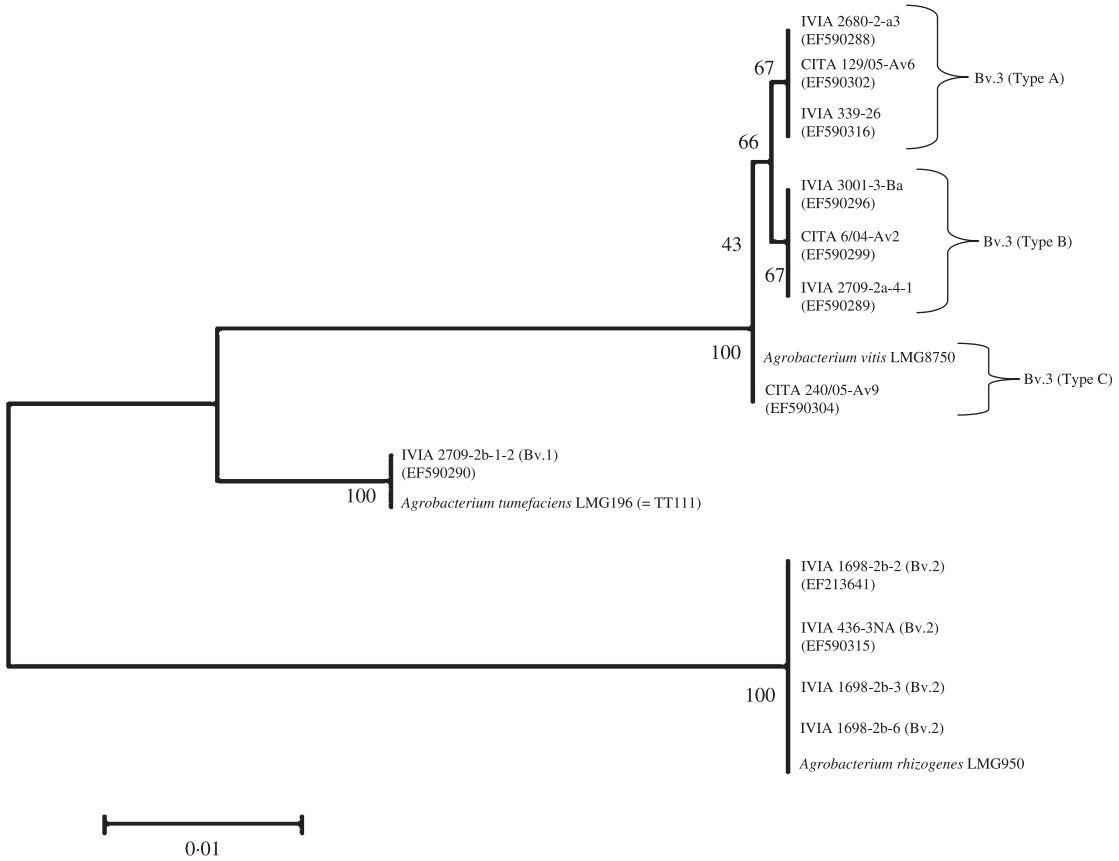

Figure 1 Dendrogram showing the relationship among strains belonging to *Agrobacterium* spp. isolated from grapevine tumours. Neighbour-joining phylogram based on 500-bp-long partial 16S rRNA from representative strains and other known related strains from GenBank. The significance of each branch is indicated by a bootstrap value calculated for 1000 subsets. The accession number of the 16S rRNA gene sequences deposited in the GenBank database are indicated in parentheses.

partial 16S rRNA gene sequence found in *A. vitis* strains presented one nucleotide change in the same position. They were 99·8–100% similar to those of the *A. vitis* strain LMG 8750 from the database. The complete 16S rRNA gene sequences of the three *A. tumefaciens* strains from two different samples were identical to each other, and showed 100% similarity to those of *A. tumefaciens* strains CFBP 2714 and LMG 196 (= TT111). Thus, according to Portier *et al*. (2006), they could belong to the genomic species G1 of *A. tumefaciens*. The complete 16S rRNA gene sequences from three selected strains of *A. vitis* type A differed from each other in one or two nucleotides (data not shown). In contrast, in both types B and C of *A. vitis* strains, three extended complete sequences of the 16S rRNA gene were identical within each type (data not shown).

PCR amplifications

PehA primers yielded the expected 199-bp amplified fragment from the coding region of the pectin enzyme hydrolase gene with the DNA from the 50 *A. vitis* strains (Table 2). No amplification band was obtained either with DNA from the three strains identified as *A. tumefaciens* or with the three *A. rhizogenes* strains. The expected amplified fragment was also obtained with DNA from the *A. vitis* reference strain, whereas no amplification was obtained with the DNA from reference strains of other biovars (Table 2). However, a fragment of different size (*c*. 500 bp) was obtained from DNA of *A. tumefaciens* strain C58 and its derivative Ti-plasmid-cured strain (NT1) when using these primers (Table 2).

VirA primers gave the expected amplified product of 480 bp with 37 out of the 50 *A. vitis* strains (74%) (Table 2). DNA from two *A. tumefaciens* strains from sample 2709 amplified when using these primers, but DNA from strain USC 316 did not. No amplification band was obtained with DNA from the three *A. rhizogenes* strains. The *A. vitis* reference strain yielded the expected amplified product, whereas no amplification product was obtained with DNA from any of the *A. tumefaciens* or *A. rhizogenes* reference strains.

Tmr primers gave the expected amplified product of 170 bp with DNA of 37 out of the 50 *A. vitis* strains (74%) (Table 2). DNA from two *A. tumefaciens* strains (the same ones that amplified with the virA primers) and the three *A. rhizogenes* strains also yielded the expected amplified product (Table 2).

VirB/G and VCF/VCR primers did not amplify DNA from *A. vitis* or *A. tumefaciens* strains (Table 2); only DNA from the three *A. rhizogenes* strains gave the expected 246-bp and 730-bp products, respectively. The virB/G primer set yielded the expected product with the nopaline-type *A. tumefaciens* and *A. rhizogenes* reference strains, whereas no amplification was obtained with the DNA from the octopine-type *A. vitis* or *A. tumefaciens* reference strains. The VCF/VCR primer set yielded the expected 730-bp amplified product with DNA from pathogenic *A. tumefaciens* and *A. rhizogenes* reference strains, but not with DNA from the *A. vitis* reference strain.

Interestingly, a correlation was observed in all *A. vitis* strains between the amplification results (positive or negative) obtained with the virA and tmr primer sets. Overall, only two different PCR amplification profiles were found for all of them. Curiously, the pathogenic *A. tumefaciens* octopine-type strain from sample USC 316 did not amplify with any of the Ti-plasmid-encoded primer sets.

Association between chromosomal (16S rRNA gene sequence) and Ti plasmid (PCR amplifications) features in A. *vitis* strains

The 14 strains of *A. vitis* showing the 16S rRNA sequence type A gave PCR profile types + −−−− with the primers pehA, virA, tmr, vir B/G and VCF/VCR, respectively, in 12 instances, and + + + −− in two instances. All type-B sequences (20 strains) showed the profile + + + −−; and those of sequence type C (16 strains) gave the profiles + + + −− and + −−−− in 19 and one instance(s), respectively. Within each sample from which several strains of *A. vitis* were analysed (samples 2739, 3105, 2709, 103/5, 3001, 44/06 and 240/5), all strains of a given type of 16S rRNA gene sequence showed only one type of profile (+ + + −− or + −−−−) (Table 2). Within samples in which several strains of *A. vitis* showed only one type of 16S rRNA gene sequence, only one profile was found. The profile + + + −− correlated with *A. vitis* strains able to use octopine. The profile + −−−− correlated with strains that did not utilize either opine, except for one *A. vitis* strain from sample 103/05.

Discussion

Tumorigenic *Agrobacterium* strains belonging to biovars 1, 2 and 3 were isolated from Spanish grape plants showing crown and/or cane galls. As expected, the majority of Spanish strains were biovar 3, indicating that the disease on grapevines is mainly caused by *A. vitis* strains, in agreement with results obtained in other countries (Burr & Otten, 1999). All *A. vitis* strains were tumorigenic on grape and also on tobacco plants, suggesting that tobacco is a suitable host for pathogenicity tests of *A. vitis* strains from Spain. A few strains belonged to biovars 1 and 2. The *A. tumefaciens* strains found on grapevines were atypical of biovar 1, because they utilized L-(+)-tartrate as the sole carbon source, probably because of the transfer of the pTAR plasmid from *A. vitis* strains (Gallie *et al*., 1984; Szegedi *et al*., 1992; Otten *et al*., 1995). Degradation of this compound by *A. tumefaciens* strains might be the result of their adaptation to grapevines, as postulated for *A. vitis* (Salomone *et al*., 1998). In fact, the biovar 1 strains were found together with *A. vitis* in the same galled plant and all probably contained the same octopine Ti plasmid. The PCR profile obtained with the octopine Ti plasmid of these *A. tumefaciens* strains isolated from sample 2709 was similar to that found with octopine Ti plasmids from *A. vitis* strains, but not to that of the octopine *A. tumefaciens* reference strain. Szegedi *et al*. (2005) reported that *A. tumefaciens* grapevine strains contained four types of Ti plasmid and, as in the present study, tartrate utilization was exclusively associated with an octopine/cucumopine-*A. vitis*-type Ti plasmid, but not with other types of Ti plasmid. All the data suggest that the *A. tumefaciens* strains from grape here contained an octopine-type Ti plasmid typical for *A. vitis* strains, this plasmid conferring a limited host range when present in *A. tumefaciens* strains. These *A. tumefaciens* strains were non-pathogenic on tomato and tobacco, in contrast to *A. vitis* isolated from the same plants and *A. tumefaciens* reference strains. A further host-range study on other hosts confirmed their limited host range, which is unusual in biovar 1 strains. On the other hand, although *A. rhizogenes* strains were sometimes isolated from grapevine galls (Panagopoulos *et al*., 1978; Süle, 1978), their existence still constitutes a rare event. [Note that the strain Ag28 (CFBP 1905) reported by Ridé *et al*. (2000), which they indicated was a biovar 2 strain isolated from grape, was actually isolated from *Prunus dulcis* (M. Fischer-Le Saux, personal communication)].

Agrobacterium vitis strains can be classified into four main groups based on opine utilization and hybridization with probes from Ti plasmids (Paulus *et al*., 1989). The first group includes those strains harbouring octopine-cucumopine Ti plasmids containing a small region of T and A bases encoding for utilization of both corresponding opines. The second group is composed of strains harbouring octopine-cucumopine Ti plasmids with a large TA region encoding for catabolism of these opines and also for that of nopaline in most cases. The third group contains strains harbouring nopaline-type plasmids, and the fourth group includes a few strains harbouring vitopine plasmids (Paulus *et al*., 1989). Based on this classification, Spanish *A. vitis* strains might contain three out of the four types of Ti plasmids, and most of the strains probably fell into the first group, because they utilized octopine, but not nopaline. These strains probably harbour highly related octopine-cucumopine Ti plasmids, because all of them (except one) showed the same PCR amplification profile. Ti plasmids from only three strains may have belonged to the second group, because they utilized both octopine and nopaline. Some *A. vitis* strains did not utilize either analysed opine, so they may have belonged to the fourth group (vitopine-type strains) or have been users of other opines. A previous opine utilization study with some Spanish *A. vitis* strains also found these three kinds of

opine users (López *et al*., 1988). Curiously, the Ti plasmid from *A. vitis* strain CITA 103/05-Av4 shared features in common with the first Paulus *et al*. (1989) group of Ti plasmids, because it was an exclusive octopine-utilizing strain, and also with the third group, according to its PCR profile (Table 2). Consequently, it could represent a rare natural transconjugant. This characterization study of Ti plasmids from *A. vitis* agrees with previous results obtained in other countries (Ma *et al*., 1987; Paulus *et al*., 1989; Ridé *et al*., 2000), although the present study did not find any *A. vitis* strain harbouring a plasmid able to catalyse only nopaline.

Partial 16S ribosomal RNA gene sequences clearly clustered strains belonging to the three species, confirming their biovar classification. Partial sequences for the few *A. tumefaciens* strains were identical to each other; the same was true among the *A. rhizogenes* strains. In contrast, partial sequences for *A. vitis* strains could be differentiated into three types, but still clearly clustered together (Sawada *et al*., 1993). The 16S rRNA gene sequence analyses in the present study reinforced the results of previous studies where *A. vitis* strains were divided into chromosomal groups by restriction enzyme analysis of the PCR-amplified 16S-23S rRNA intergenic region (Otten *et al*., 1996b). Complete 16S rRNA gene sequences of the three *A. tumefaciens* isolated from two different vineyard areas were identical. In contrast, complete 16S rRNA gene sequences for representative strains of each of the three *A. vitis* types (A, B and C) differed from each other by one or two nucleotides at the same position. The complete 16S rRNA gene sequence of *A. vitis* strain S4 also differed from the K309 type strain sequence by one nucleotide (Otten *et al*., 1996a).

The pehA primers yielded the corresponding amplified fragment with DNA from all *A. vitis* strains, but not with DNA from *A. rhizogenes* or *A. tumefaciens* strains, confirming that this primer set can be used as universal for *A. vitis* detection (Eastwell *et al*., 1995). Curiously, *A. tumefaciens* C58 and NT1 (C58 strain cured of Ti plasmid) reference strains also yielded an amplification product of a different size from that of the corresponding pehA-amplified fragment. Strain C58 may contain an ortholog for the pectin enzyme hydrolase (*pehA*) gene, or the obtained amplicon was an unspecific product. DNA from most of the *A. vitis* Spanish strains amplified with virA primers (Eastwell *et al*., 1995), as did the DNA of two *A. tumefaciens* stains. Surprisingly, a positive correlation was found between amplification with these primers and the results obtained with tmr primers, in both species. VirB/G and VCF/VCR primer sets only amplified DNA from *A. rhizogenes* strains, not DNA from *A. tumefaciens* or *A. vitis* strains. With respect to the VirB/G primers, Nesme *et al*. (1989) found them specific for the nopaline Ti plasmid, which agrees with the present results, where only the *A. rhizogenes* strains contained exclusively nopaline Ti plasmids. With respect to the VCF/VCR primers, Sawada *et al*. (1995) and Cubero *et al*. (1999) found that these sequences were common to most of the *A. vitis* strains. However, Szegedi & Bottka (2002) described the failure of VCF/VCR primers for detection of *A. vitis* strains, with only one out of 16 strains amplified, and in the present study none were amplified. In the study by Sawada *et al*. (1995), most of the VCF/VCR positive *A. vitis* strains were isolated in Japan, but, in contrast when analysing strains from other countries, only one out of three amplified. Cubero *et al*. (1999) analysed only five *A.vitis* strains, four of them being isolated from the same sample. As none of the primer pairs assayed so far allowed the universal detection of all possible pathogenic *Agrobacterium* species found in grapevine tumours, a first step for screening could be the use of both pehA and tmr primer pairs for detection of *Agrobacterium* spp. strains in grapevine plant material. This is a useful result of the study and in fact, when 60 samples from tumours from naturally infected grapevines were analysed by PCR and bacterial isolations (using common and selective media), detection using both pehA and tmr primer pairs was more efficient than bacterial isolation (unpublished data). However, further investigations are needed for the design of an accurate and reliable protocol for detecting only pathogenic *Agrobacterium* spp. strains in grapevine samples.

Three different Ti plasmid types were found among *A. vitis* strains, based on PCR amplifications and opine utilization profiles, and three different types of 16S rRNA gene sequence were found. In all analysed samples, each chromosomal background contained only one type of Ti plasmid, showing a strong association between chromosomal backgrounds and Ti plasmids within each sample. This fact might indicate a clonal structure of the population with an apparent lack of transfer between strains within the same sample. This study clearly reinforces the hypothesis of a relationship between *A. vitis* strains and their Ti plasmids (Otten *et al*., 1996b).

The increase of crown and cane galls in vineyards in Mediterranean countries could be related to exchanges of plant material and favourable conditions for disease development. This study provides the first characterization of a systematic collection of *Agrobacterium* spp. strains isolated from Spanish vineyards. Such characterization will greatly contribute to the knowledge of the causal agent of crown gall disease on grapevines in the Mediterranean area. Moreover, the combined use of two PCR primer pairs employed in this study allowed the identification of the three *Agrobacterium* species found on grapevines.

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References

Anonymous, 1993. *Journal Officiel des Communautés Européenes*. No. L 250/4-8 (7/10/93).

Argun N, Momol MT, Maden S *et al*., 2002. Characterization of *Agrobacterium vitis* strains isolated from Turkish grape cultivars in the Central Anatolia region. *Plant Disease* **86**, 162–6.

Burr TJ, Bazzi C, Süle S, Otten L, 1998. Crown gall of grape: biology of *Agrobacterium vitis* and the development of disease control strategies. *Plant Disease* **82**, 1288–97.

Burr TJ, Otten L, 1999. Crown gall of grape: biology and disease management. *Annual Review of Phytopathology* **37**, 53–80.

Cangelosi GA, Best EA, Martinetti G, Nester EW, 1992. Methods for genetic analysis of *Agrobacterium*. *Methods in Enzymology* **204**, 384–97.

Cubero J, López MM, 2001. An efficient microtitre system to determine *Agrobacterium* biovar. *European Journal of Plant Pathology* **107**, 757–60.

Cubero J, Martínez MC, Llop P, López MM, 1999. A simple and efficient PCR method for the detection of *Agrobacterium tumefaciens* in plant tumours. *Journal of Applied Microbiology* **86**, 591–602.

Dessaux Y, Petit A, Farrand SK, Murphy MP, 1998. Opine and opine-like molecules involved in plant-*Rhizobiae* interactions. In: Spaink HP, Kondorosi A, Hooykans P, eds. *The Rhizobiaceae*. Dordrecht, the Netherlands: Kluwer Academic Publishers, 173–97.

Eastwell JC, Willis LG, Cavileer TD, 1995. A rapid and sensitive method to detect *Agrobacterium vitis* by polymerase chain reaction. *Plant Disease* **79**, 822–7.

Gallie DR, Zaitlin D, Perry KL, Kado CI, 1984. Characterization of the replication and stability regions of *Agrobacterium tumefaciens* plasmid pTAR. *Journal of Bacteriology* **157**, 739–45.

Haas JH, Zueibil A, Zutra D, Tanne E, Manulis S, 1991. The presence of crown gall of grape incitated by *Agrobacterium tumefaciens* biovar 3 in Israel. *Phytoparasitica* **19**, 311–8.

Keane PJ, Kerr A, New PB, 1970. Crown gall of stone fruit. II. Identification and nomenclature of *Agrobacterium* isolates. *Australian Journal of Biological Sciences* **23**, 151–3.

Knauf VC, Panagopoulos CG, Nester EW, 1983. Comparison of the Ti plasmid from three different biotypes of *Agrobacterium tumefaciens* isolated from grapevines. *Journal of Bacteriology* **153**, 1535–42.

Lastra B, 1998. Agrobacterium *spp. en Cultivares de Vid de Galicia: Distribución, Caracterización de Cepas Bacterianas y Sensibilidad de Cultivares y Portainjertos.* Santiago de Compostela, Spain: University of Santiago de Compostela, PhD thesis.

López MM, 2004. Tumores de la vid (*Agrobacterium* spp. (Smith y Townsend) (Conn). In: *Los Parásitos de la Vid. Estrategias de Protección Razonada*. Madrid, Spain: Ministerio de Agricultura, Pesca y Alimentación: Mundi-Prensa Editions, 245–7.

López MM, Bertolini E, Olmos A *et al*., 2003. Innovative tools for detection of plant pathogenic viruses and bacteria. *International Microbiology* **6**, 233–43.

López MM, Gorris MT, Montojo AM, 1988. Opine utilization by Spanish isolates of *Agrobacterium tumefaciens*. *Plant Pathology* **37**, 565–72.

Ma D, Yanofsky MF, Gordon MP, Nester EW, 1987. Characterization of *Agrobacterium tumefaciens* isolates from grapevine tumors in China. *Applied and Environmental Microbiology* **53**, 1338–43.

Martínez-Murcia AJ, Antón AI, Rodríguez-Valera F, 1999. Patterns of sequence variation in two regions of the 16S rRNA multigene family of *Escherichia coli*. *International Journal of Systematic Bacteriology* **49**, 601–10.

Mohammadi M, Fatehi-Paykani R, 1999. Phenotypical characterization of Iranian isolates of *Agrobacterium vitis*, the causal agent of crown gall disease of grapevine. *Vitis* **38**, 115–21.

Moore LW, Kado CL, Bouzar H, 1988. *Agrobacterium*. In: Schaad NW, ed. *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. St Paul, MN, USA: APS Press, 16–36.

Nesme X, Leclerc MC, Bardin R, 1989. PCR detection of an original endosymbiont: the Ti plasmid of *Agrobacterium tumefaciens*. In: Nardon P, Gianinazzi-Peason V, Greines AM, Margullis L, Smith C, eds. *Endocitobiology*, **IV edn**. Paris, France: INRA, 47–50.

New PB, Kerr A, 1971. A selective medium for *Agrobacterium radiobacter* biotype 2. *Journal of Applied Bacteriology* **34**, 233–6.

Ophel K, Kerr A, 1990. *Agrobacterium vitis* sp. nov. for strains of *Agrobacterium* biovar 3 from grapevines. *International Journal of Systematic Bacteriology* **40**, 236–41.

Otten L, Crouzet P, Salomone JY, de Ruffray P, Szegedi E, 1995. *Agrobacterium vitis* strain AB3 harbors two independent tartrate utilization systems, one of which is encoded by the Ti plasmid. *Molecular Plant-Microbe Interactions* **8**, 138–46.

Otten L, de Ruffray P, de Lajudie P, Michot B, 1996a. Sequence characterization of a ribosomal RNA operon from *Agrobacterium vitis*. *Molecular and General Genetics* **251**, 99–107.

Otten L, de Ruffray P, Momol EA, Momol MT, Burr TJ, 1996b. Phylogenetic relationship between *Agrobacterium vitis* isolates and their Ti plasmids. *Molecular Plant-Microbe Interactions* **9**, 782–6.

Panagopoulos CG, Psallidas PG, Alivizatos AS, 1978. Studies on biotype 3 of *Agrobacterium radiobacter* var. *tumefaciens*. In: *Proceedings of the Fourth Conference on Plant Pathogenic Bacteria*. Angers, France 221–8.

Paulus F, Huss B, Bonnard G *et al*., 1989. Molecular systematics of biotype III of Ti plasmids of *Agrobacterium tumefaciens*. *Molecular Plant-Microbe Interactions* **2**, 64–74.

Portier P, Fischer-Le Saux M, Mougel C *et al*., 2006. Identification of genomic species in *Agrobacterium* biovar 1 by AFLP genomic markers. *Applied and Environmental Microbiology* **72**, 7123–31.

Ridé M, Ridé S, Petit A, Bollet C, Dessaux Y, Gardan L, 2000. Characterization of plasmid-borne and chromosome-encoded traits of *Agrobacterium* biovar 1, 2 and 3 strains from France. *Applied and Environmental Microbiology* **66**, 1818–25.

Roy MA, Sasser M, 1983. A medium selective for *Agrobacterium tumefaciens* biotype 3. *Phytopathology* **73**, 810.

Salomone JY, Szegedi E, Cobanov P, Otten L, 1998. Tartrate utilization genes promote growth of *Agrobacterium* spp. on grapevine. *Molecular Plant-Microbe Interactions* **11**, 836–8.

Sawada H, Ieki H, Masuda I, 1995. PCR detection of Ti and Ri plasmids from phytopathogenic *Agrobacterium* strains. *Applied and Environmental Microbiology* **61**, 828–31.

Sawada H, Ieki H, Oyaizu H, Masuda I, 1993. Proposal for rejection of *Agrobacterium tumefaciens* and revised descriptions for the genus *Agrobacterium* and for *Agrobacterium radiobacter* and *Agrobacterium rhizogenes*. *International Journal of Systematic Bacteriology* **43**, 694–702.

Schroth MN, Thompson JP, Hildebrand DC, 1965. Isolation of *Agrobacterium tumefaciens-Agrobacterium radiobacter* group from soil. *Phytopathology* **55**, 645–7.

Süle S, 1978. Biotypes of *Agrobacterium tumefaciens* in Hungary. *Journal of Applied Bacteriology* **44**, 207–13.

Szegedi E, Bottka S, 2002. Detection of *Agrobacterium vitis* by polymerase chain reaction in grapevine bleeding sap after isolation on a semiselective medium. *Vitis* **41**, 37–42.

Szegedi E, Bottka S, Mikulás J, Otten L, Sule S, 2005. Characterization of *Agrobacterium tumefaciens* strains isolated from grapevine. *Vitis* **44**, 49–54.

Szegedi E, Otten L, Czakó M, 1992. Diverse types of tartrate plasmids in *Agrobacterium tumefaciens* biotype III strains. *Molecular Plant-Microbe Interactions* **5**, 435–8.

Zhu J, Oger PM, Schrammeijer B, Hooykaas PJJ, Farrand SK, Winans SC, 2000. The bases of crown gall tumorigenesis. *Journal of Bacteriology* **182**, 3885–95.

Zupan J, Muth TR, Draper O, Zambryski P, 2000. The transfer of DNA from *Agrobacterium tumefaciens* into plants: a feast of fundamental insights. *The Plant Journal* **23**, 11–28.